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## Original

# Bone Regeneration by Low-dose Recombinant Human Bone Morphogenetic Protein-2 Carried on Octacalcium Phosphate Collagen Composite

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Abstract: Bone morphogenetic protein-2 (BMP-2) has diverse functions and is especially important in bone and cartilage development. Recombinant human BMP-2 (rhBMP-2) is an osteoinductive growth factor that has been clinically applied as a bone graft substitute. However, high-dose rhBMP-2 can cause complications such as induction of significant swelling that can endanger the patient's life. Atelocollagen sponge (ACS) is the commercially provided standard carrier of rhBMP-2 in clinical applications. However, a large concentration of rhBMP-2 is required to be clinically effective with ACS as the carrier. Octacalcium phosphate/collagen (OCP/Col) has been shown to be an excellent bone substitute compared with other bone substitute materials such as hydroxyapatite or β-tricalcium phosphate due to its biological properties. In this study, we evaluated the use of OCP/Col as a carrier to minimize the effective dose of rhBMP-2. ACS or OCP/Col discs impregnated with different rhBMP-2 concentrations were implanted in mice calvarial bone defects. Morphological analysis with micro-CT both at 4 and 6 weeks post-implantation showed homogenous hard tissue formation in the defects of the OCP/Col group at all rhBMP-2 concentrations tested (0, 0.25, 0.50, or 1.00 μg). In contrast, ACS alone or with 0.25 μg of rhBMP-2 showed almost no bone formation. However, bone mineral density in all groups of ACS and OCP/Col was not dependent on rhBMP-2 concentration. Histological evaluation indicated that bone formation progressed depending on rhBMP-2 concentration in the defects of both the ACS and OCP/Col groups, although the newly formed bone area was significantly higher in the OCP/Col group than in the ACS group. These results indicate that OCP/Col could be an effective carrier of rhBMP-2, minimizing the application dose of rhBMP-2 in clinical settings and avoiding the complications caused by high-dose rhBMP-2.

Key words: Bone regeneration, Carrier, Collagen, Octacalcium phosphate, Bone morphogenetic protein-2

## Introduction

Bone regeneration is challenging in oral and maxillofacial surgery because there are many cases that require bone regeneration in this region. In current clinical settings, autologous bone grafting remains the gold standard for bone regeneration of jawbone defects because of its superior osteoinductivity and osteoconductivity. However, this technique has several disadvantages such as limited availability and donor site morbidity. Hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) have been widely applied in clinical settings[1-3)]; however, these materials have not replaced autogenous bone grafts in light of their superior biocompatibility and osteoconductivity but not osteoinductivity[4)]. The clinical application of recombinant human bone morphogenetic protein-2 (rhBMP-2), which is one of the most important bone inductive proteins, has been expected, and it has been approved for clinical use in extraction socket preservation and maxillary sinus floor augmentation[5)]. rhBMP-2 shows superior bone regeneration, however, several adverse events have been reported such as local edema, seroma, and cancer induction at high-dose rhBMP-2[6-8)]. Atelocollagen sponge (ACS) is presently used as a carrier of rhBMP-2; however, it is reported to release rhBMP-2 instantaneously[9)]. Accordingly, it is necessary to identify a carrier that will spontaneously release rhBMP-2 and can act as a bone substitute[10)].

Octacalcium phosphate (OCP) is a direct precursor of biological apatite, which sustainably and irreversibly converts into biological apatite under physiological conditions[11)]. Moreover, OCP has been demonstrated to enhance osteoblastic cell differentiation[12)] and is effective for bone regeneration because of its high bone regenerative ability and rapid absorbability compared with HA or β-TCP[13)]. OCP possesses many desirable properties as a bone substitute; however, it cannot be molded using sintering processes because of its crystal structure. In order to improve its handling property, a composite comprising OCP and collagen (OCP/Col) was developed. OCP/Col was shown to yield significantly enhanced bone regeneration compared with β-TCP or HA collagen composite[14)]. OCP/Col has also been shown to be both safe and efficacious in human tooth extraction sockets, cystic cavities, and maxillary sinus floor elevation[15-18)]. BMP-2 is known to absorb calcium phosphate and

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also collagen[19, 20]. Therefore, we propose that OCP/Col could be a superior carrier for rhBMP-2 to minimize the required rhBMP-2 dose. The objective of the present study is to assess the osteogenic potential of low-dose rhBMP-2 carried on OCP/Col compared with that carried on ACS.

## Materials and Methods

### *Preparation of OCP, OCP/Col, atelocollagen, and their combination with/without rhBMP-2*

OCP was synthesized by direct precipitation as previously described[21], and sieved granules (particle size: 300–500 μm) were obtained. Collagen solution was prepared from NMP collagen PS (Nippon Meat Packers, Tsukuba, Ibaraki, Japan), a lyophilized powder of pepsin-digested atelocollagen isolated from porcine dermis. Collagen was dissolved in distilled water and adjusted to 3% of the final concentration at pH 7.4. Then, sieved OCP granules were added to the collagen solution and mixed. The proportion of OCP in OCP/Col was adjusted to 77 weight%. This mixture was then lyophilized and molded into discs (diameter: 9 mm, thickness: 1 mm). OCP discs then underwent dehydrothermal treatment (150°C, 24 h) in a vacuum drying oven and sterilization using gamma-ray irradiation (5 kGy). The resultant discs were punched to 5 mm in diameter using a HANDY PUNCH KIT (product number: 3H-PS10, H.H.H. Manufacturing Co., Osaka, Japan). rhBMP-2 (produced by INFUSE® Bone Graft, Medtronic, Memphis, TN, USA) was dissolved in distilled water at concentrations of 0, 0.01, 0.02, or 0.04 μg/μl. Then, 25 μl of rhBMP-2 solution was dripped onto the OCP/Col or ACS disc (control group; diameter: 5 mm, thickness: 1 mm). These materials were defined as OCP/Col, 0.25 OCP/Col, 0.50 OCP/Col, 1.00 OCP/Col, ACS, 0.25 ACS, 0.50 ACS, and 1.00 ACS.

### *Animals and implantation procedures*

All animal experiments were performed at the Nagasaki University Animal Experiment Facility and were in accordance with protocols approved by the Local Institutional Animal Care and Use Committee of Nagasaki University (approval no. 1606211320). Eighty 10-week-old male C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan) were used. Mice were housed in standard rodent cages in a light- and temperature-controlled room in the Biomedical Research Center, Center for Frontier Life Sciences, Nagasaki University. Animals had free access to standard laboratory chow and tap water. Transplantation experiments were conducted under general anesthesia. General anesthetics were prepared by mixing 1.875 ml of medetomidine hydrochloride (1 mg/ml), 2 ml of midazolam (5 mg/ml), 2.5 ml of butorphanol (5 mg/ml), and 18.625 ml of physiological saline to yield a final liquid volume of 25 ml. The mixture was then subcutaneously or intraperitoneally injected at a concentration of 0.1 ml/10 g body weight. After disinfection of the operative field, an arc-shaped skin incision was made from the left to right preauricular region via the forehead region. The periosteum of the calvarium was ablated and a full-thickness standardized trephine defect, 5 mm in diameter, was made in the calvarium under continuous saline buffer irrigation. Each material was then implanted into the trephine defect. Five mice were treated per group. After the defects were treated, the ablated periosteum and skin were repositioned and sutured. For the OCP/Col and ACS groups, the tissues were collected at four and six weeks postoperatively.

### *Morphological and quantitative analysis by micro-computed tomography*

The morphological and quantitative image analysis of newly formed bone was performed using micro-computed tomography (CT) [micro X-ray CT machine for laboratory animals (R mCT), RIGAKU, Tokyo, Japan] under standardized conditions (90 kV, 150 mA, two minutes). In three-dimensional analysis using structural analysis software (TRI/3D-BON, RATOC System Engineering, Osaka, Japan), the newly formed bone area was analyzed after sampling using bone mineral density (BMD) standards, of established densities of between 300 mg/cm$^3$ and 1,500 mg/cm$^3$, and the extracted range of bone was defined between 300 mg/cm$^3$ and 1,100 mg/cm$^3$.

### *Tissue preparation and histological examination*

After micro-CT examination, samples were immersed in PBS overnight and fixed in 4% paraformaldehyde for 24 h. Samples were then decalcified in EDTA for 10 days and washed with distilled water. Thereafter, samples were cut coronally into two pieces at the center of the defect and embedded in paraffin. Serial sections 5 μm thick were cut coronally and stained with hematoxylin and eosin. Images were taken using a photomicroscope (Axiocam ERc 5s, ZEISS, Oberkochen, Germany).

### *Quantitative measurement of cortical bone and bone marrow area*

The amount of cortical bone and bone marrow area in the implanted material was measured from the histological sections prepared near the center of the defect. The cortical bone and bone marrow data was extracted using Adobe Photoshop® CS6 Extended. After converting to JPEG images, the data volume (Kilobytes) of cortical bone and bone marrow was measured with Windows 10 photo viewer.

### *Statistical analysis*

Statistical analysis of all micro-CT data was performed using JMP® version 13 (SAS Institute, Cary, NC, USA). The temporal change of each OCP/Col group's BMD value was analyzed by a nonparametric multiple comparison test. Values are expressed as mean ± standard deviation (SD). A p-value less than 0.05 was statistically significant.

## Results

### *Morphological analysis by micro-CT*

Micro-CT analysis revealed that the ACS and 0.25 ACS groups showed almost no hard tissue formation, whereas the 0.50 ACS and 1.00 ACS groups showed hard tissue formation. The formation area was slightly wider in the 1.00 ACS group at both 4 and 6 weeks postoperatively compared with the 0.50 ACS group. In contrast, hard tissue formation was recognized within the defect in the OCP/Col groups at all rhBMP-2 concentrations, and the radio-lucent area was decreased rhBMP-2 dose-dependently (Fig. 1).

### *Morphometric analysis of newly formed bone volume by micro-CT analysis*

The OCP/Col groups produced a greater volume of newly formed bone than the ACS groups. Namely, 0.25 OCP/Col, 0.50 OCP/Col, and 1.00 OCP/Col showed significant increases in newly formed bone volume compared with 1.00 ACS, which showed the highest bone volume among the ACS groups, at four weeks, while 1.00 OCP/Col showed significant increases in bone volume compared with 1.00 ACS at 6 weeks (Fig. 2).

### *Bone mineral density (BMD)*

BMD tended to increase from 4 to 6 weeks postimplantation in all groups. There were also no significant differences in BMD between the ACS and OCP/Col groups. For example, BMD of 1.00 OCP/Col, which

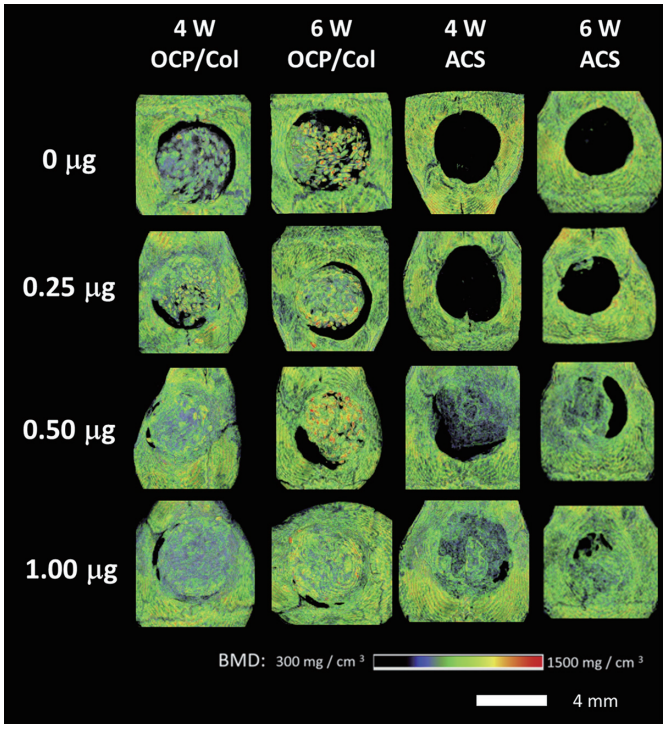


Figure 1. Tissue formation in the ACS and OCP/Col groups with and without rhBMP-2 at 4 and 6 weeks postoperatively. The ACS and 0.25 ACS groups showed almost no hard tissue formation, whereas the 0.50 ACS and 1.00 ACS groups showed hard tissue formation. The formation area was slightly wide in the 1.00 ACS group at both 4 and 6 weeks postoperatively. In contrast, uniform hard tissue formation was recognized within the defect in the OCP/Col groups at all rhBMP-2 concentrations.

produced the highest bone volume, was not significant different compared with ACS alone both at four and six weeks (Fig. 3).

***Histological evaluation***

ACS and 0.25 ACS showed no bone formation in the defect, whereas 0.50 and 1.00 ACS showed a certain amount of new bone formation at four weeks. On the other hand, in the OCP/Col groups, the area of new bone gradually increased as the concentration of rhBMP-2 increased (Fig. 4). ACS and 0.25 ACS showed slight bone formation in the defect, and 1.00 ACS demonstrated bone bridging of the defect with new bone at six weeks. In contrast, the OCP/Col group showed remarkable osteogenesis compared with the ACS group (Fig. 5). Almost all implanted ACS was absorbed in all ACS groups, while OCP/Col remained, especially at the lower concentrations of rhBMP-2. Additionally, newly formed bone in the OCP/Col groups was thicker than that in the ACS groups. Although OCP/Col without rhBMP-2 showed new bone formation, OCP/Col with rhBMP-2 showed more mature bone formation with bone marrow.

***Quantitative measurement of cortical bone and bone marrow area***

The area of newly formed cortical bone and bone marrow area measured using histological specimens showed a similar tendency, that is, bone volume measured with micro-CT increased in a rhBMP-2 dose-dependent manner for both the ACS and OCP/Col groups. The area of the OCP/Col groups was higher than that of the ACS groups at the respective concentrations of rhBMP-2. Statistically significant differences were observed between the lower (0, 0.25) and higher (0.50, 1.00) concentrations of rhBMP-2 in both the ACS and OCP/Col groups (Fig. 6). 0.25 OCP/Col, which was the lowest concentration of rhBMP-2, showed similar bone area with 0.50 and 1.00 ACS at 6 weeks.

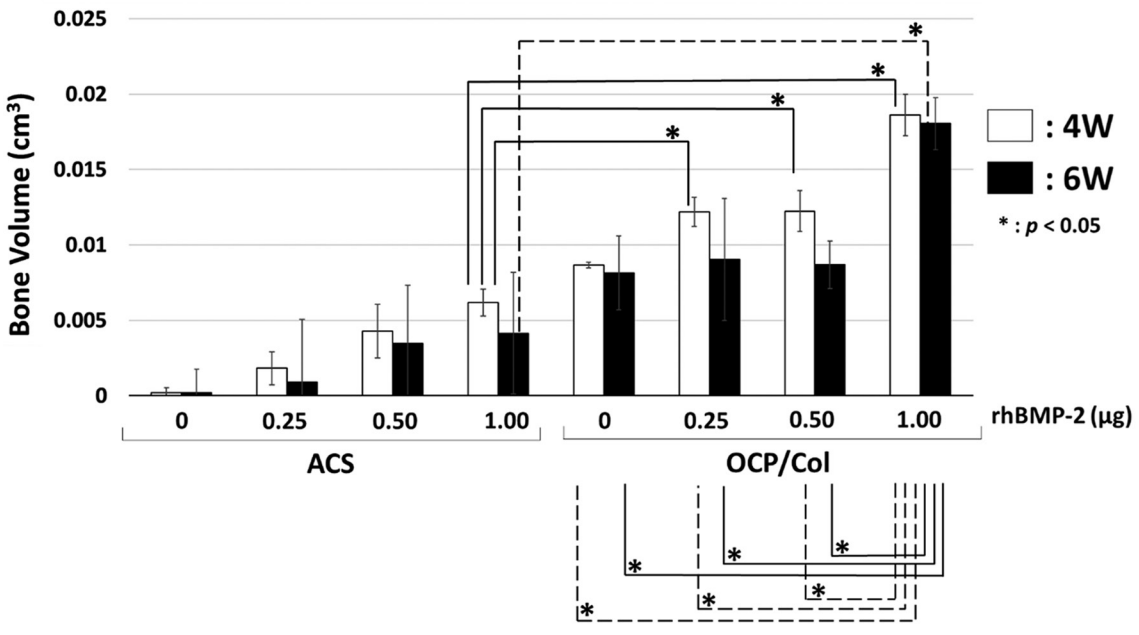


Figure 2. Bone volume in the ACS and OCP/Col groups with and without rhBMP-2 at 4 and 6 weeks postoperatively. At 4 weeks, 0.25 OCP/Col, 0.50 OCP/Col, and 1.00 OCP/Col showed significant increases in newly formed bone volume compared with 1.00 ACS. At 6 weeks, 1.00 OCP/Col showed significant increases in bone volume compared with 1.00 ACS, whereas, OCP/Col, 0.25 OCP/Col, and 0.50 OCP/Col did not. * : $p<0.05$

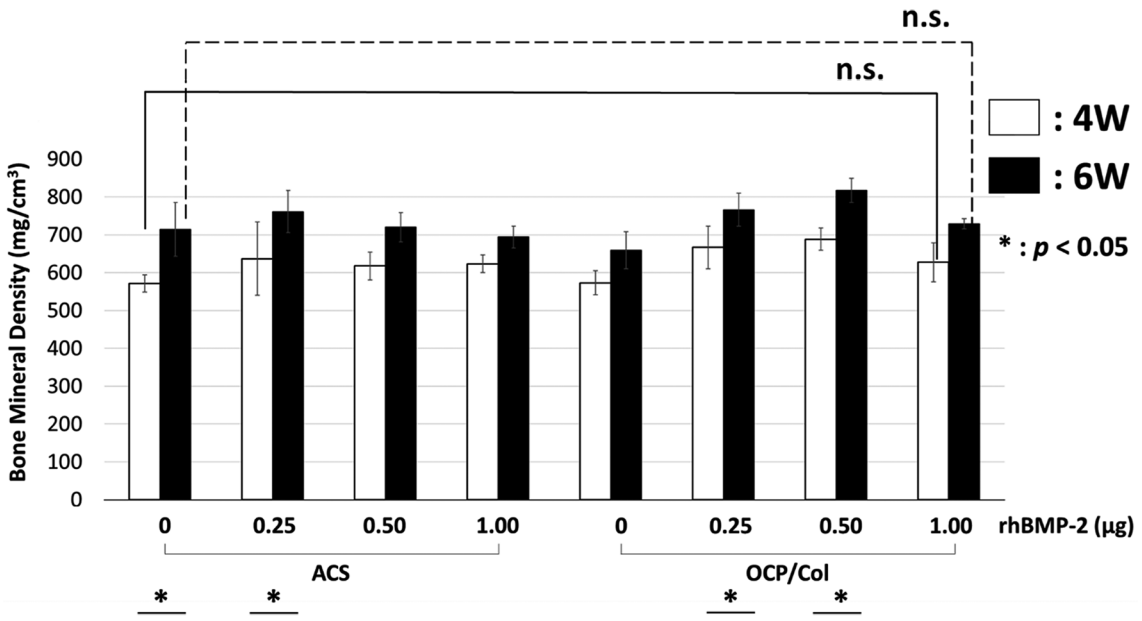


Figure 3. Bone mineral density in the ACS and OCP/Col groups with and without rhBMP-2. At both 4 and 6 weeks postoperatively, the bone mineral density of 1.00 OCP/Col with rhBMP-2 was not significantly different compared with ACS without rhBMP-2. * : p<0.05

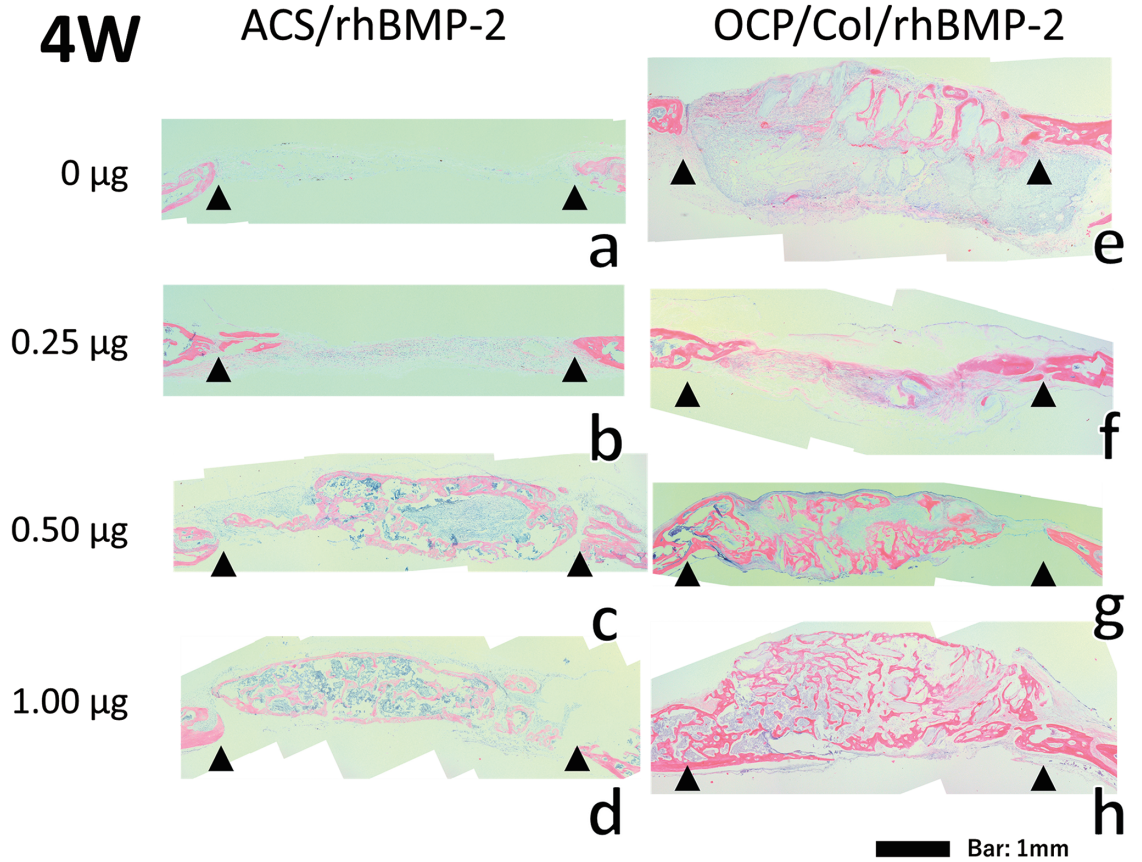


Figure 4. Effect of ACS and OCP/Col with and without rhBMP-2 on bone formation in defects at 4 weeks postoperatively. At 4 weeks ACS and 0.25 ACS showed no bone formation in the defect, whereas 0.50 and 1.00 ACS showed slight new bone formation. On the other hand, in the OCP/Col groups, the area of new bone gradually increased as the concentration of rhBMP-2 increased.

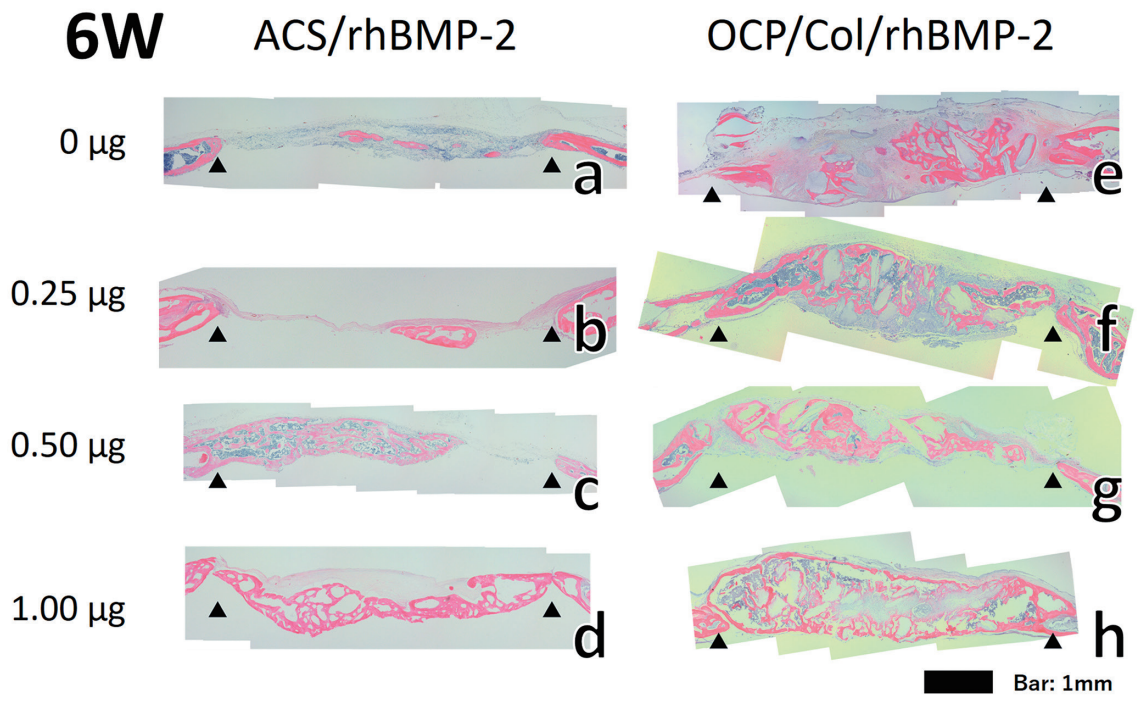


Figure 5. Effect of ACS and OCP/Col with and without rhBMP-2 on bone formation in defects at 6 weeks postoperatively. At 6 weeks the ACS and 0.25 ACS groups showed slight formation in the defect, and the 1.00 ACS group showed bone bridging of the defect with new bone. In contrast, the OCP/Col group showed remarkable osteogenesis compared with the ACS group. Although OCP/Col without rhBMP-2 showed remarkable new bone formation, OCP/Col with rhBMP-2 showed more mature bone formation with bone marrow. Additionally, newly formed bone in the OCP/Col groups was thicker than that in the ACS groups.

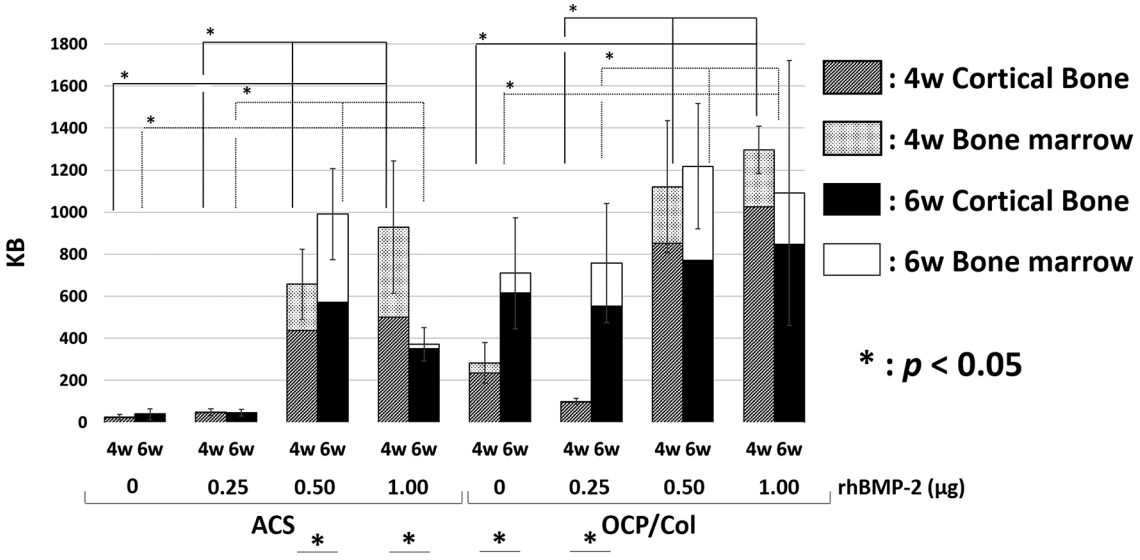


Figure 6. Effect of ACS and OCP/Col with and without rhBMP-2 on areas of cortical bone and bone marrow at 4 and 6 weeks postoperatively. The areas of cortical bone and bone marrow were increased in an rhBMP-2 dose-dependent manner in both the ACS and OCP/Col groups, and the OCP/Col groups showed higher bone areas than the ACS groups. Statistically significant differences were observed between the lower (0, 0.25) and higher (0.50, 1.00) concentrations of rhBMP-2 in both the ACS and OCP/Col groups. * : $p<0.05$

## Discussion

In the current study, we examined the potential application of OCP/Col as an rhBMP-2 carrier to minimize the effective dose of rhBMP-2 on bone regeneration. Our findings demonstrate that low-concentration rhBMP-2 impregnated on OCP/Col discs could induce sufficient bone formation. According to the qualitative analysis using micro-CT and histological findings, the concentration of rhBMP-2 could be decreased to less than one-fourth using OCP/Col as the carrier compared with ACS (Figs. 2 and 6). In terms of clinical application of BMP, selection of an appropriate carrier is extremely important for the following reasons. Since rhBMP-2 diffuses within a short time when topically administered without a carrier[22], an appropriate carrier for proper delivery, retention,

and sustained released of BMP-2 is needed[19]. The carrier must be nontoxic, noncarcinogenic, nonantigenic, proinflammatory, and preferably biodegradable and moldable[23]. There are four major categories of rhBMP-2 carriers: natural-origin polymers, inorganic materials, synthetic biodegradable polymers, and composites[24]. In current clinical settings, ACS is the commercially provided standard carrier for rhBMP-2 among the presently available carriers that fulfill the requirements described above[25]. However, a high rhBMP-2 concentration is required when ACS is used as the carrier[17]. As previously demonstrated, high doses of BMP-2 cause severe clinical side effects such as ectopic bone formation, osteoclast-mediated bone resorption, inappropriate adipogenesis, and cancer[19, 26]. Additionally, high-dose rhBMP-2 can induce acute edema and swelling, leading to fatal airway obstruction in the oral and maxillofacial region[6, 27]. Therefore, low-dose rhBMP-2 should be applied to avoid these adverse effects. In the present clinical settings, the concentration of rhBMP-2 with ACS as a carrier is 37.5 μg/μl. Even taking into an account the differences between humans and mice in the response to BMP, it is expected that the concentration of rhBMP-2 can be reduced when OCP/Col is used as a carrier.

There are a number of reasons why OCP/Col is a potential superior carrier for rhBMP-2 compared with ACS. First, in accordance with a previous report, OCP/Col itself has bone inductive activity[28]. As shown in Figs. 1, 4 and 5, new bone formation was observed apart from the rim of the bone defect in the OCP/Col only group (without rhBMP-2), indicating that OCP/Col shows osteoconductivity as well as osteoinductivity. It is apparent that the bone inductive activity of rhBMP-2 augments that of OCP/Col, since OCP/Col with rhBMP-2 induced more mature bone formation with cortical bone and bone marrow compared with OCP/Col without rhBMP-2 (Figs. 1, 4 and 5). BMD tended to increase time-dependently, but significant differences were not observed. There were also no significant differences among the ACS and OCP/Col groups with different concentrations of rhBMP-2 (Fig. 3). This suggests that while the quality of bone is similar, the quantity is different between ACS and OCP/Col as carriers. Second, the composition of OCP/Col is favorable for use as an rhBMP-2 carrier because rhBMP-2 is known to bind tightly to calcium phosphate and collagen, which are major components of OCP/Col[19, 20]. It is speculated that rhBMP-2 is released as OCP and collagen are degraded, allowing the slow release of rhBMP-2[26, 27]. Remnants of OCP/Col were observed at 6 weeks postimplantation, while ACS was completely absorbed. This might assist in the sustainable release of rhBMP-2 with OCP/Col as the carrier. Furthermore, rhBMP-2 influences chemotaxis and mesenchymal stem cells proliferation and differentiation into osteoblasts, thus, the porous structure of OCP/Col, composed of collagen, is a suitable environment for cell migration[29]. Lastly, OCP/Col has superior mechanical strength to ACS[30]. We observed that newly formed bone in the OCP/Col implant group was thicker than that in the ACS implant groups (Figs. 4 and 5). This finding suggests that OCP/Col is sufficiently strong to resist pressure from the skin flap. rhBMP-2/ACS has been approved for clinical application for socket preservation and sinus floor augmentation in the oral and maxillofacial region[31]. In these areas, the implant material does not suffer from pressure because it is surrounded by the bone wall. However, the implant material needs to have certain mechanical strength in the case of alveolar bone ridge augmentation. In this respect, rhBMP-2 with OCP/Col could be a good implant material for guided bone regeneration (GBR) because it possesses both strength and flexibility.

In the present study, we demonstrated superior bone regeneration of rat calvarial bone defects with rhBMP-2 carried on OCP/Col. Although OCP/Col itself has bone inductive activity, low-dose rhBMP-2 enhances this activity. The minimization of the effective dose of rhBMP-2 reduces its side effects. Recently, there has been increasing demand for alveolar bone regeneration in response to the popularization of implant dentistry. We suggest that rhBMP-2 with OCP/Col could be an ideal implant material for GBR because of its superior osteoinductivity and mechanical properties such as strength and flexibility. In conclusion, though OCP/Col itself is a good bone substitute, it could also be an effective carrier for rhBMP-2, thereby reducing the effective dose of rhBMP-2 and resulting in its safe clinical application for more reliable bone augmentation.

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**Conflict of Interest**

The authors have declared that no COI exists.

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